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COMPARISON OF API 20E AND PCR FOR IDENTIFICATION OF *SALMONELLA*

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Abstract API 20E and *invA* PCR were compared for diagnostic accuracy for *Salmonella* for 310 bacterial isolates from 3 Illinois swine farms. Reactions based on Triple Sugar Iron agar, Lysine Iron Agar, and *Salmonella* O (poly A/B) antisera tests were also considered. Repetitive sequence PCR (rep-PCR) using REP, BOX, and ERIC primers, identified the genetic basis for diagnostic classification. Cluster analysis and multidimensional scaling grouped isolates based on diagnostic and genetic characteristics. The *invA* PCR had higher agreement with other tests (particularly poly A/B antisera) than API 20E in *Salmonella* classification. Cluster analysis identified several clusters of isolates that were API 20E positive but negative by other tests, suggesting lower specificity for API 20E than *invA* PCR. Rep-PCR genotyping supported a genetic basis for diagnostic test result differences. This suggests that *invA* PCR should be considered as a cost and time saving alternative to API 20E in the diagnosis of *Salmonella*.

Introduction Isolation and accurate identification of *Salmonella* is a significant challenge in clinical microbiology (Hoorfar *et al*, 1999; McDonough *et al*, 2000; Perry *et al*, 2002). Among the commercially available identification systems, API 20E, which relies on biochemical substrate utilization for classification, has often been used as the standard of comparison for identification of members of the family Enterbacteriaceae (O'Hara *et al*, 1992; Overman *et al*, 1985; Koneman *et al*, 1997). However, API 20E has not always yielded satisfactory results (Robinson *et al*, 1995; Rutherford *et al*, 1977; Aldridge *et al*, 1981). Rather than relying on phenotypic traits, identification of bacteria such as *Salmonella* could be improved by recognition of genotypic characteristics, e.g., by using polymerase chain reaction (PCR) techniques targeting gene sequences unique to Salmonella (Vaneechoutte and Van Eldere, 1997). The purpose of the present study is to compare API 20E with a PCR for detection of the *invA* gene (Chiu and Ou, 1996), within the context of other diagnostic tests, using multivariate statistical methods to evaluate isolate classification and diagnostic test accuracy.

Materials and Methods Fecal and floor samples (1 gm) were collected from 3 Illinois swine farms, each visited twice over a 6 month period during the winter and spring of 2003. Two farms were farrow-to-finish operations and the third was a feeder pig finisher operation. Pigs were kept mostly in total confinement facilities.

Samples were placed directly into tetrathionate broth (9 ml for fecal, 25 ml for floor samples) and transported to the laboratory, where they were incubated at 37°C. After 48 hr, 100 μl of each sample was transferred to 10 ml Rappaport-10 broth and incubated at 37°C. After 24 hr, 10 μl of broth was plated onto a Xylose-Lysine-Tergitol-4 (XLT$) agar plate and incubated at 37°C. After 24 hr, 1 or 2 bacterial colonies with *Salmonella* morphology (red with black centers) were selected from each plate, subcultured onto brilliant green agar plates (BGA), and incubated at 37°C. After 24 h, 1 colony with typical *Salmonella* morphology (red/pink color) was selected from each BGA plate and streaked onto a tryptic soy agar plate (TSA). TSA plates were incubated for 24 h at 37°C, then held at 4°C for further testing.

Template DNA for PCR amplification was prepared by adding a small number of cells from a single TSA plate colony to 100 μl sterile Millipore water and boiling for 5 minutes. An *inv-A* PCR was also performed using an oligonucleotide primer set producing a 244 bp amplicon from the *invA* gene of *Salmonella*, as described in Chiu and Ou (1996). Amplicons were identified using agarose gel electrophoresis.

An API 20E strip was inoculated for each isolate and incubated at 37°C for 24 hr. Positive results were evaluated for each of the 20 biochemical tests and diagnosis of *Salmonella* determined using the API 20E Analytical Profile Index (July 1999 edition) or by calling the API Voice Response System.

Additional diagnostic tests were performed on each isolate to validate API 20E and invA PCR test results and account for disagreements between the tests. Slide agglutination tests were performed using Salmonella O Antisera Poly A and Poly B. Triple sugar iron (TSIA) and lysine iron agar

(LIA) slants were inoculated with each isolate and incubated at 37°C for 24 hr. An oxidase test was also performed on each isolate.

Genotyping using repetitive sequence PCR (Rep-PCR) with REP, BOX, and ERIC primers (Weigel *et al.*, 2004), was performed for each sample (samples had either 1 or 2 isolates evaluated with *Salomonella* diagnostics), randomly selecting 1 member of pair if 2 isolates were evaluated per sample. Genetic distances between samples were calculated based on fragment size matching patterns, with results from the 3 primers combined to calculate a 3D Euclidean distance (ibid.). Complete linkage hierarchical cluster analysis using the 3D distance matrix was conducted to determine the genetic basis for diagnostic test result differences.

The agreement in the classification of isolates as *Salmonella* between *invA* PCR and API 20E at the 70%, 85%, and 90% *Salmonella* likelihood levels, and each of the aforementioned with the agglutination and the individual biochemical tests, including each of the 20 API 20E composite tests, was determined by calculating kappa values (Cohen, 1969).

In order to evaluate agreement among tests using the composite of test results, multivariate data analysis was conducted. Classification of isolates into distinct groups based on test result differences was accomplished using cluster analysis (Anderberg, 1973) and multidimensional scaling (Kruskal and Wish, 1978). The variables selected for the multivariate analyses were those that contributed to the variability observed between isolates; variables giving redundant results were eliminated. Similarity among isolates over all diagnostic test results was calculated using a simple matching coefficient. An initial hierarchical cluster analysis using the complete linkage algorithm was conducted to estimate the number of diagnostic groups. Multidimensional scaling, using the matrix of isolate matching coefficients, projected cases into multidimensional space and provided spatial coordinates as input for centroid sorting cluster analysis, which then classified cases into diagnostic groups. For each classification group, the percentage positive for each diagnostic test was then calculated to characterize the group in terms of *Salmonella* identity.

Results There were 310 suspected *Salmonella* isolates obtained in this study, of which 279 (90.0%) were identified by API 20E as *Salmonella sp.* with likelihood values ranging from 62.5% to 99.9%. There were 214 isolates (69.0%) positive by *invA* PCR, all of which were API 20E positive. The 65 isolates that were API 20E positive and PCR negative had *Salmonella* likelihood values ranging from 62.5% to 98.4%. For poly A/B antisera, 219 of the isolates (70.6%) were positive. In addition, for TSIA 233 (75.1%) and LIA 264 (85.2%) isolates showed typical *Salmonella* biochemical responses.

In evaluating the agreement between API 20E at 3 *Salmonella* likelihood levels and *invA* PCR with the 20 component API tests and other diagnostic tests, API 20E had highest agreement ($\kappa \geq 0.8$) with other tests at the 85% likelihood value: LIA lysine decarboxylase (LDC) and the API-LDC component ($\kappa = 0.93$), followed by invA PCR ($\kappa = 0.86$), poly A/B antisera ($\kappa = 0.84$), and the API ornithine decarboxylase (ODC) component ($\kappa = 0.83$). The invA PCR had the highest agreement with poly A/B antisera ($\kappa = 0.94$), API-ODC ($\kappa = 0.93$), API-LDC ($\kappa = 0.91$), and LIA-LDC ($\kappa = 0.91$). LIA-LDC and API-LDC were in complete agreement and will be referred to only as 'LDC' below. These tests will be considered further in evaluating the relative accuracy of API 20E versus invA PCR.

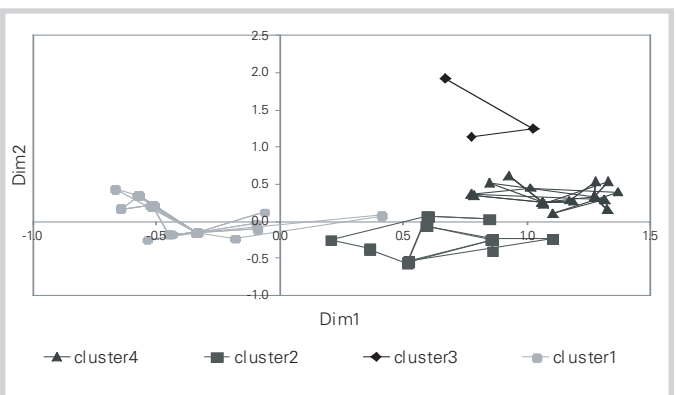

Figure 1. Classification of isolates on multidimensional scaling axed according to centroid sorting cluster group.

The initial hierarchical cluster analysis resulted in 4 main clusters; thus, centroid cluster analysis assumed the existence of 4 diagnostic groups of isolates. A satisfactory multidimensional scaling (MDS) configuration was achieved with two dimensions (Stress1 = 0.04). Figure 1 depicts the distribution of isolates in 2-dimensional (2D) space, with a superimposed minimum spanning tree (Gower and Ross, 1969) connecting

isolates within the same centroid sorting cluster analysis group. The 4 groups were distinctly separated in the 2D MDS space.

The 218 isolates in cluster 1 were all classified by API 20E as Salmonella (likelihood ? 85%), by invA PCR as Salmonella in all but 3 cases, and as Salmonella in all but 2 cases by poly A/B antisera, LDC, and ODC. The 58 isolates in cluster 2 were all invA PCR negative, but included 14 isolates that were API 20E positive; there were also 9 isolates positive by LDC, but only 1 positive each by ODC and poly A/B antisera. Of the 3 isolates in cluster 3, 0 were positive by API 20E, invA PCR, and poly A/B antisera, 1 was LDC positive, and 2 were ODC positive. Of the 31 isolates in cluster 4, 0 were positive by invA PCR and LDC, 1 was API 20E positive, 2 were poly A/B antisera positive, and 5 were ODC positive. Given this association of diagnostic test results with cluster membership, it is apparent from Figure 1 that MDS dimension 1 represents invA PCR, with positive isolates negative on this axis; dimension 2 represents to some degree the distinction between API 20E positive and negative test results, with the negative API 20E isolates (particularly clusters 3 and 4) clearly positive on this axis.

There were 172 samples available for rep-PCR genotyping. A schematic representation of the dendrogram for the cluster analysis of similarity of rep-PCR banding patterns is depicted in Figure 2. There were 4 clusters separated by genetic similarity values > 20%. The first cluster consisted of 125 samples that were all positive by API 20E (≥ 85% likelihood), invA PCR, poly A/B antisera, and LDC. Clusters 2 and 3, more closely linked to each other than to the other clusters, consisted of 7 samples positive by API 20E and negative by the other tests represented. Cluster 4 consisted of 40 samples that were all *invA* PCR and poly A/B antisera negative, but for which 20% of samples were positive by API 20E and LDC.

Discussion/Conclusions The API 20E diagnostic test for *Salmonella*, based on identifying the biochemical properties of the bacteria, has been the standard test for *Salmonella* detection in many diagnostic laboratories. The comparison of API to other *Salmonella* diagnostics has revealed that API 20E classifies more isolates as *Salmonella* than do other tests. Compared to API 20E, the higher agreement of the *invA* PCR to the poly A/B antisera and API-ODC tests, as well as nearly equal agreement to LDC suggest that API 20E has a higher false positive rate. The highest agreement of API 20E with other tests was achieved at the 85% likelihood level; agreement was lower at lower and higher likelihood levels.

The genetic separation by rep-PCR of a portion of the API positives indicates an underlying genetic basis for the disagreement of API 20E and *invA* PCR, and further supports the conclusion that API 20E has lower diagnostic specificity for classification of bacterial isolates as *Salmonella*. Given this, the absence of samples that were *invA* PCR positive and API 20E negative suggests that both tests have approximately equal sensitivity in detecting *Salmonella*.

The agglutination test using poly A/B antisera had high

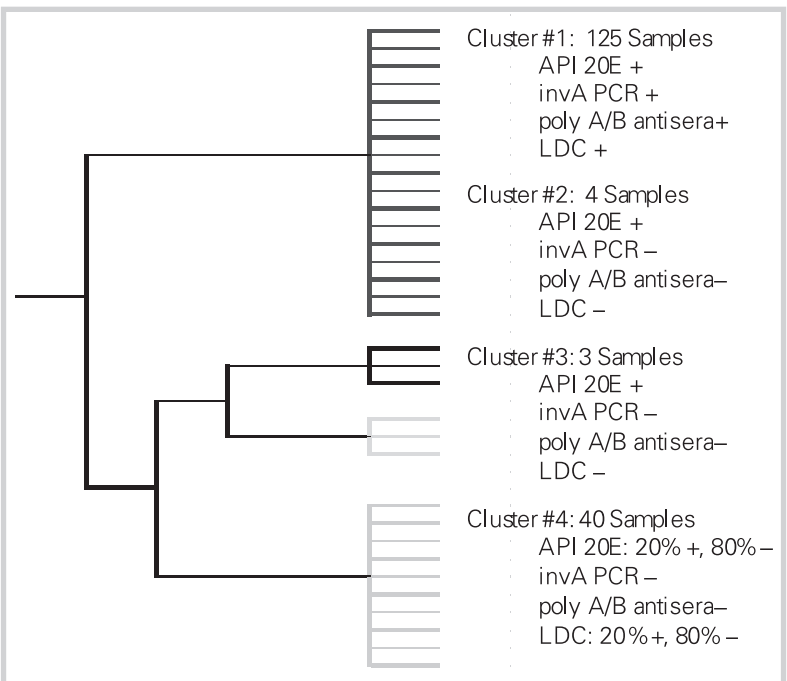

Figure 2. Schematic representation of clusters of *Salmonella* isolates as determined by complete linkage, based on genetic distances from 3 primers (REP, BOX, ERIC) of repetitive sequence PCR.

agreement (κ = 0.94) with *invA* PCR and clustered in a nearly identical pattern in the centroid clustering based on diagnostic test results, as well as in the hierarchical clustering based on rep-PCR genetic classification. This strengthens the validity of *invA* PCR in differentiating bacterial isolates based on Salmonella specific characteristics.

The results of this study need to be considered with in the context of the samples evaluated. The bacteria cultured were from the environment of swine production systems. There were only 3 farms evaluated in a localized geographic region, during cold (winter) to moderate (spring) temperatures. Results could differ in other environments and thus the generalizability of the the present findings need to be evaluated.

The API 20E test is more expensive (about $6 per sample) than the *invA* PCR (< $2 per sample). Thus, *invA* PCR is a cost effective alternative to API 20E, once PCR equipment such as a thermal cycler and electrophoretic gel apparatus are available. The *invA* PCR also produces results in less time ($\approx$ 4 hrs, compared to $\approx$ 16 hrs for API 20E). Thus, in laboratories equipped for PCR, the *invA* test for *Salmonella* detection should be considered as an alternative to API 20E.

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